Modulation of the Alveolar Macrophage Superoxide Production by Protein Phosphorylation

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Stimulation of alveolar macrophages (AM) with adenosine-5-diphosphate (ADP) results in transient production of superoxide anion radical ($O_2^{\cdot-}$; superoxide) and H_2O_2 in a metabolic event known as the respiratory burst. Initiation of the respiratory burst appears to depend on activation of protein kinase activity, whereas protein phosphatases might involved in termination of the burst. The involvement of protein kinase C was suggested by inhibition by bisindolylmaleimide I (GF 109203X), a relatively specific inhibitor. KN-62, an inhibitor of calcium–calmodulin protein kinase II, also partly inhibited the respiratory burst stimulated by ADP and phorbol esters. The role of protein phosphatases in termination of the ADP-stimulated respiratory burst of AM was examined with calyculin A (CA) (25–75 nM) or okadaic acid (OA) (1–5 µM), two inhibitors of protein phosphatase 1 and 2a (PP1;PP2a). A dose-dependent prolongation of the respiratory burst was observed in the presence of these inhibitors. CA and OA also markedly enhanced the rate of superoxide production stimulated by ADP, consistent with involvement of PP1/PP2a in regulating both the rate of activation and timing of termination. Treatment of AM with cyclosporin A (CsA) (1–50 µM), an inhibitor of the calcium-dependent protein phosphatase 2b (PP2b), stimulated superoxide production by itself and significantly prolonged the duration of ADP-stimulated superoxide production. CsA, however, did not increase the ADP-stimulated rate of superoxide production. Thus, PP1/PP2a appear to be the primary phosphatases for controlling the intensity of the respiratory burst during receptor-elicited superoxide production in AM, whereas PP1/PP2a and PP2b play a role in turning off the respiratory burst. — Environ Health Perspect 106(Suppl 5):1185–1190 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-5/1185-1190forman/abstract.html

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Introduction

Phagocytes such as neutrophils (PMN) and alveolar macrophages (AM) generate superoxide anion radicals ($O_2^{\cdot-}$; superoxide) transiently when stimulated by a variety of particulate or soluble agents (1,2). This process, also called the respiratory burst, plays an important role in host defense against microbial infections and is the result of the activation of an NADPH oxidase that catalyzes the one-electron reduction of oxygen at the expense of NADPH. H_2O_2 is then produced by spontaneous dismutation of $O_2^{\cdot-}$. The NADPH oxidase is a multicomponent enzyme that is dormant in unstimulated cells and composed of cytosolic and membrane components. The assembly of the oxidase has been extensively studied using a cell-free system where the NADPH-dependent formation of superoxide is induced by a detergent such as sodium dodecyl sulfate (SDS) or lipids such as arachidonic acid and others (3–5). Upon stimulation, the cytosolic components of the oxidase (i.e., p47^{phox}, p67^{phox}, and p40^{phox}, and rac1/rac2) translocate to the membrane where they join with intrinsic membrane components (i.e., gp91^{phox} and p22^{phox}, the two subunits of the flavocytochrome b to form the catalytically active NADPH oxidase (6,7). The mechanisms by which assembly occurs are still largely unknown; however, there is increasing evidence for a requirement for phosphorylation in intact cells. Stimulation with various agents results in an increase in the phosphorylation on serine, threonine, and tyrosine of many proteins in neutrophils. Among them, p47^{phox} has consistently been shown to become extensively phosphorylated on multiple serine residues (8–11). Several studies demonstrated that this multiple phosphorylation is an essential step in intact PMN and is required for translocation of this protein and for activation of the respiratory burst oxidase but is dispensable in the cell-free system (12,13). Although many studies have focused on the mechanisms of activation, less is known about the turnoff mechanisms. The duration of the respiratory burst is stimulus dependent and desensitization of the receptor by phosphorylation or other means is probably part of the deactivation of the oxidase. In addition, several studies in neutrophils suggested that dephosphorylation of components of the oxidase, in particular p47^{phox}, could also be involved. This was based on the ability of inhibitors of the protein phosphatase 1 (PP1) and protein phosphatase 2a (PP2a) to modulate the respiratory burst oxidase and the phosphorylation of p47^{phox} (14–17).

Like most types of macrophages, AM are long-lived cells and must be able to repetitively mount a respiratory burst. Thus, the termination mechanism is a vital function for these cells; however, little is known about this mechanism in macrophages. The timing of termination

of the respiratory burst in AM depends upon the type of stimulant used to initiate O_2^- production, as has been observed with PMN. We recently demonstrated that adenosine-5-diphosphate (ADP) stimulates the respiratory burst of rat AM (18). Superoxide production is initiated within seconds of addition of ADP and terminates quickly within a few minutes. This is in contrast to the delayed but extended production of superoxide stimulated by phorbol esters. The signaling pathways that are triggered by ADP in rat AM have not been extensively studied. We previously showed that treatment with ADP induces the production of inositol triphosphate (IP_3) (19), suggesting that phospholipase C, which catalyzes the production of IP_3 and diacylglyceride (DAG) is activated by the putative purinergic receptors. In agreement with these results, ADP induces a transient release in intracellular calcium (18). Furthermore, the ADP-stimulated burst is reduced in the absence of extracellular calcium or in cells pretreated with thapsigargin to deplete the IP_3-releasable calcium pool (18,20). In this study, we used pharmacologic tools to determine the role of protein phosphorylation in the activation of the NADPH oxidase by ADP.

Materials and Methods

Materials

Cytochrome c, superoxide dismutase (SOD), buffer salts, dimethylsulfoxide (DMSO), phorbol-12-myristate-13-acetate (PMA), and ADP were purchased from Sigma Chemical Co. (St. Louis, MO) Calyculin A (CA), okadaic acid (OA), KN-62, and bisindolylmaleimide I [GF 109203X (BIM)] were purchased from Calbiochem (San Diego, CA). Stock solutions were prepared in DMSO, stored in small aliquots, and appropriately diluted just before use.

Preparation of Alveolar Macrophages

AM were prepared from specific pathogen- and viral-free 250 to 300 g, Sprague-Dawley rats (CH:CD(SD)BR; Charles River, Inc., Wilmington, MA). Animals were sacrificed by intraperitoneal injection of pentobarbital (100 mg/kg), and the AM were obtained by pulmonary lavage with 100 ml 0.01 M sodium phosphate-buffered saline (14). The pelleted AM were suspended in Krebs-Ringer-phosphate buffer ([KRPH], pH 7.4, containing 10 mM HEPES, 125 mM NaCl, 5 mM KCl,

1 mM MgSO_4, 10 mM sodium phosphate, 5 mM glucose, 1.3 mM CaCl_2) and stored at 4°C until used. This preparation yields approximately 10^7 cells per rat, of which greater than 97% are viable AM.

Measurement of Alveolar Macrophage Respiratory Burst

AM (1×10^6 cells/ml) were incubated in 1 ml KRPH buffer with DMSO ($\leq 0.1\%$) or with the appropriate dilution of the inhibitors for 15 min at 37°C. The respiratory burst was triggered by the addition of stimulus (100 ng/ml PMA or 100 µM ADP). Superoxide production was measured by a continuous assay as the SOD-inhibitable reduction of ferricytochrome c, followed as a function of time at 550 to 540 nm in a dual wavelength spectrophotometer (Shimadzu UV-3000, Kyoto, Japan). Under these conditions, DMSO did not affect the respiratory burst.

Results

### Role of Protein Kinases in the ADP-Stimulated Respiratory Burst

Our previous studies indicated that ADP stimulation induces production of IP_3 (19) and the release of calcium from intracellular stores (18). A rise in calcium concentration can activate two types of calcium-dependent protein kinases, the classical protein kinases C (cPKCs) and the calcium–calmodulin kinase II (CAMP-KII). DAG activates both cPKCs and the novel PKCs (nPKCs). In addition, removal of extracellular calcium, treatment with verapamil (a calcium channel blocker), and depletion of the IP_3-releasable calcium pool by pretreatment with thapsigargin (18,20), each significantly reduced the ADP-stimulated burst (18), indicating that calcium-dependent pathways play a role in the activation of the NADPH oxidase by ADP. To probe the role of PKCs pharmacologically, we used various concentrations of BIM, a cell-permeant analog of staurosporine that in $vitro$ inhibits more specifically classical and nPKCs at low concentrations (10 nM) (21). As expected, the respiratory burst, stimulated by an optimal concentration of PMA (100 ng/ml), was inhibited by BIM with an IC_50 of 2 µM (Figure 1) and reached complete inhibition at 30 µM. BIM also inhibited the respiratory burst stimulated by 100 µM ADP, although the IC_50 (5.8 µM) was higher and complete inhibition was never obtained, even at 100 µM BIM. Nevertheless, these data suggest

that PKC participates in the activation of the NADPH oxidase by ADP.

The effects of KN-62 on the ADP-stimulated burst were also tested. KN-62 inhibits the kinase activity of CAMPKII by binding to the calmodulin-binding site of the enzyme with an in $vitro$ IC_50 of 0.9 µM. As seen in Figure 2, KN-62 induced a slight inhibition of both the ADP- and PMA-stimulated respiratory burst with a similar dose response. Incubation with 10 µM KN-62 resulted in 49 and 56% inhibition of the PMA- and ADP-stimulated respiratory bursts, respectively.

Role of Protein Phosphatases in the ADP-Stimulated Respiratory Burst

Protein phosphorylation is a dynamic process and is the result of a balance between the activity of kinases and phosphatases.
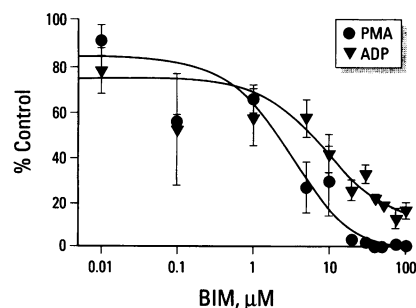
Figure 1. Inhibition of the respiratory burst by BIM. AM were incubated with the indicated concentrations of BIM for 15 min. Superoxide production was measured by a continuous assay after stimulation with PMA or ADP, as described in" Materials and Methods." The mean ± SE for each dose is shown (PMA n=4; ADP n=7). BIM alone did not stimulate superoxide production.
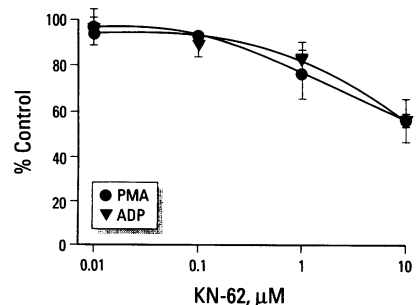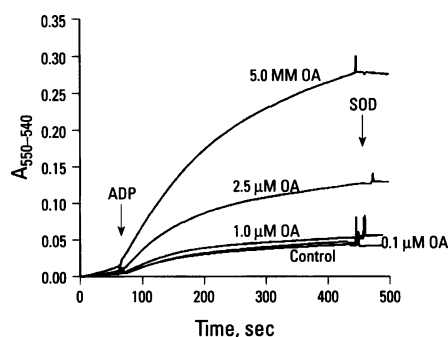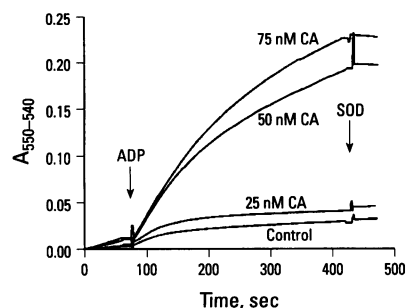

Figure 2. Inhibition of the respiratory burst by KN-62. AM were incubated with the indicated concentrations of KN-62 for 15 min. Superoxide production was measured by a continuous assay after stimulation with PMA or ADP as described in "Materials and Methods." The mean ± SE for each dose is shown (PMA n=3; ADP n=7). KN-62 did not stimulate superoxide production.

Although the role of kinases in the activation of the NADPH oxidase has been extensively studied, less is known about the role of serine–threonine phosphatases. Recently, new pharmacologic agents were developed as useful tools to determine the role of these phosphatases in a biologic response. OA is produced by several types of marine plankton and is a potent inhibitor of PP2a (IC_{50} 0.1 nM) and to a lesser extent of PP1 (IC_{50} 10–100 nM); while CA inhibits both PP1 and PP2a with approximately equal potency (IC_{50} 2 and 1 nM, respectively) (22). Incubation of AM with 0.1 and 1 mM OA had minimal effects on the ADP-stimulated respiratory burst; however, higher concentrations of OA resulted in an enhancement in superoxide anion radical release (Figure 3). CA similarly enhanced

Figure 3. Enhancement of superoxide production by okadaic acid. AM were incubated with the indicated concentrations of OA for 15 min. Superoxide production was measured by a continuous assay before and after stimulation with ADP as described in "Materials and Methods." This is representative of 3 separate experiments.

the ADP-stimulated respiratory burst, although lower doses, in the nanomolar range, were sufficient to induce an equivalent increase in superoxide release (Figure 4). CA, on its own, also induced a slight increase in cytochrome *c* reduction that was inhibited by SOD, indicating that this was due to superoxide production. OA and CA induced both an increase in the initial rate of release and a delay in the termination of the burst with an 8- to 15-fold increase in the total amount of superoxide produced at 5 min (Table 1). The ADP burst terminates within 2 min; however, in the presence of the phosphatase inhibitors, the release of superoxide by 2 min was equivalent to the initial rate and was still going on by 5 min. These data suggest that serine–threonine phosphatases play a role in the ADP-stimulated respiratory burst. The doses of OA used in the study may appear high compared to the *in vitro* IC_{50}; however, the cells were only incubated for 15 min with the inhibitor to prevent activation by warming and adhesion to the walls of the tubes. Longer incubation times might have resulted in inhibition at lower doses. The fact that OA at doses less than 1 mM has no effect is suggestive of a lesser role for PP2a in the modulation of the ADP-stimulated burst. However, we cannot be certain of the relative specificity of the two inhibitors for PP1 and PP2a *in vivo* without knowing their actual intracellular concentrations.

Protein phosphatase 2b (PP2b), also known as calcineurin, is another serine-threonine phosphatase and its activity appears to be regulated by a calcium-calmodulin complex. The calcium sensitivity of PP2b indicates that it might be involved in calcium-dependent pathways.

Recently, it was shown that the immuno-suppressant drugs cyclosporin A (CsA) and FK506 affect the activity of PP2b (23,24). Thus, these drugs could be useful pharmacologic tools to probe the role of PP2b in the activation of the NADPH oxidase. As seen in Figure 5, incubation of AM with the highest concentrations of CsA in the absence of stimulation resulted in an increase of the superoxide release that was inhibited by SOD. In addition, CsA had little effect on the initial rate of superoxide release, contrary to what was observed with OA and CA (Table 1). However, the duration of the ADP-stimulated burst similarly increased at 25 and 50 µM CsA. Thus, these data suggest that PP2b might participate in the turning off of the ADP-stimulated respiratory burst.

Discussion

Studies in intact cells have suggested that protein phosphorylation plays an important role in the activation of the NADPH oxidase. Cloning of the various components of the oxidase demonstrated that they contain SH3 domains and proline-rich regions that can interact with each other and are important in the assembly of the catalytically active oxidase at the plasma membrane (25–27). It was suggested that part of the activation process involves changes in the conformation of the various components, revealing new binding sites. Phosphorylation or detergents such as SDS could provide the means for such conformation changes (28). Since the discovery that p47[phox], one of the cytosolic components of the respiratory burst oxidase, becomes rapidly phosphorylated upon stimulation with several agents, the focus has been to

Figure 4. Enhancement of superoxide production by calyculin . AM were incubated with the indicated concentrations of CA for 15 min. Superoxide production was measured by a continuous assay before and after stimulation with ADP as described in "Materials and Methods." This is representative of 3 separate experiments.

Table 1. Effect of protein phosphatase inhibitors on superoxide production by rat alveolar macrophages.

Inhibitor	Concentration	Initial rate	Superoxide produced in 5 min
		% Control ± SE	
Okadaic acid	0.5 µM	97 ± 3	86 ± 8
	1 µM	131 ± 6	114 ± 9
	2.5 µM	323 ± 30	343 ± 42
	5 µM	486 ± 22	881 ± 87
Calyculin A	25 nM	137 ± 12	105 ± 10
	50 nM	373 ± 30	1046 ± 83
	75 nM	470 ± 76	1528 ± 79
Cyclosporin	1 µM	114 ± 8	132 ± 16
	10 µM	110 ± 6	130 ± 28
	25 µM	106 ± 21	212 ± 41
	50 µM	128 ± 16	351 ± 65

Cells were incubated with indicated concentrations of inhibitors for 15 min. Superoxide production was measured continuously after stimulation with ADP as described in" Materials and Methods." Any superoxide production stimulated by the protein phosphatase inhibitors alone was not subtracted. This is the mean % control ± SE of 3 separate experiments.
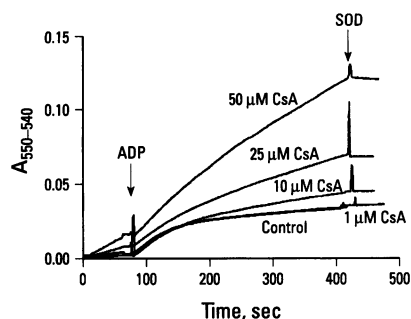
Figure 5. Enhancement of superoxide production by cyclosporin A. AM were incubated with the indicated concentrations of CsA for 15 min. Superoxide production was measured by a continuous assay before and after stimulation with ADP as described in "Materials and Methods." This is representative of 3 separate experiments.

define the kinases that phosphorylate p47^{phox} *in vivo*, which is still a matter of debate. PKC was an obvious candidate, as phorbol esters are very strong activators of the respiratory burst and induce the phosphorylation of several serines at the C-terminus of p47^{phox} within consensus phosphorylation sites for PKC (17,29,30). However, using various methodologies, several groups showed that PKC, cAMP protein kinase (PKA), the mitogen-activated protein kinases p38MAPK and ERK2, and the p21-activated kinases (PAKs) could phosphorylate p47^{phox} *in vitro* (10,31–33). In addition, phosphatidic acid can induce the phosphorylation of p47^{phox} in the cell-free system by activating a novel protein kinase, still to be identified (34). Recently, the phosphorylation of p67^{phox}, which exists in a complex with p47^{phox} in the cytosol, has been shown to occur upon stimulation in a PKC-dependent and PKC-independent manner (35). Furthermore, Park et al. (13) showed that, in a protein kinase-dependent cell-free system, the initial step of the activation process was taking place in the membrane and required the action of one or more kinases. This step might also exist in intact cells and implicate the phosphorylation upon stimulation of the two subunits of the flavocytochrome *b* that was reported several years ago by Segal and co-workers (36,37). Thus, there is compiling evidence that, independently of the various signaling pathways that might regulate the oxidase activity at the receptor level upstream of the oxidase, more than one kinase is involved in the phosphorylation of other oxidase components. In this report, we used pharmacologic tools to investigate the signaling pathways that might regulate the activation

of the respiratory burst oxidase by the adenine nucleotide ADP. KN-62, which has been described as a relatively specific inhibitor of CAMPKII, also inhibited the ADP burst at 10 µM, a concentration that inhibits 90% of the CAMPKII activity and 30% of the superoxide stimulated by cross-linking of FcγRII in neutrophils (38). Because KN-62 inhibited the PMA burst with a similar dose response might indicate that, in AM, KN-62 also inhibits a PKC isoform involved in the activation of the burst. Another alternative would be that KN-62 inhibits a kinase activated by PKC. The effect of KN-62 on kinases other than CAMPKII has not been extensively studied; thus, at this point it is unclear whether the inhibition of the ADP and PMA burst by KN-62 implies the involvement of CAMP-KII. Our data clearly suggest that BIM-inhibitable isoforms of PKC represent the major pathway leading to activation of the NADPH oxidase, as approximately 80% of the superoxide release was inhibited by BIM; however, it raises the possibility of the involvement of other kinases.

The role of protein phosphatases in the activation of the NADPH oxidase has been explored using the inhibitors of PP1 and PP2a, OA and CA. Treatment of PMN with these inhibitors induced an increase in the rate and extent of the respiratory burst stimulated by the chemotactic peptide f-Met-Leu-Phe (fMLP) (14,39). This is in agreement with our data in rat AM stimulated by ADP where these inhibitors had similar effects with a similar dose response, i.e., OA was efficient at doses > 1 µM and CA at nanomolar concentrations. The fact that they affect both the extent and the maximum production suggest that their targets are involved in both the activation and the continuation of the respiratory burst. The exact mechanism by which these phosphatases are effective is still unclear. Phosphatase activity was present in PMN cytosol that could dephosphorylate p47^{phox} and was inhibited by OA and CA (40). Therefore, it was suggested that dephosphorylation of p47^{phox} was responsible for the termination of the respiratory burst. However, other effects of OA and CA have been reported that might point to a less simplistic explanation. Treatment with CA alone resulted in the hyperphosphorylation of various proteins including p47^{phox} (14,16). CA inhibited the fMLP-stimulated activation of a group of renaturable kinases that were later identified as PAKs (32,41) and increased the fMLP-stimulated translocation of Rac2 (42), whereas OA

inhibited the translocation of p47^{phox} and p67^{phox} in PMA-stimulated PMN (37). Furthermore, the effect of these inhibitors varied with the stimulus, as the respiratory burst stimulated by PMA was either enhanced (14) or inhibited (39) in the presence of these inhibitors and the activation of the NADPH oxidase by serum-opsonized zymosan was inhibited by OA (43). Thus, it is still unclear whether the effect of PP1 and PP2a occurs directly at the level of components of the oxidase or at an upstream step in the signaling cascade.

In this report, we detected an increase in the superoxide production in the absence of stimulation with the highest doses of CA that did not occur in resting PMN. In addition, CsA had an even greater but similar effect. This suggests that phosphatases actively suppress the respiratory burst in resting cells. Interestingly, ADP stimulation after treatment with CsA was increased, but the maximal rate of superoxide release was not that different from the rate measured in control cells, suggesting that PP2b, a calcium-dependent enzyme, might be mainly involved in the termination of the ADP burst. We previously showed that changes in intracellular free calcium concentration ($[Ca^{2+}]_i$) play a dual role in the modulation of the respiratory burst in rat AM (44). Both internal stores and extracellular calcium contribute to the initiation of the respiratory burst stimulated by ADP (18). The PMA-stimulated respiratory burst does not require a change in $[Ca^{2+}]_i$ and superoxide production continues for much longer than the ADP burst. The simultaneous addition of a receptor-mediated stimulus with PMA results in a supra additive enhancement of the initial rate of superoxide production but terminates within the same time period as the receptor-mediated respiratory burst alone (44). These effects have been closely correlated with the time course of calcium mobilization by the receptor-mediated agonist. The addition of the calcium ionophore A23187 to cells stimulated with PMA in the presence of extracellular calcium causes a rapid decrease in superoxide production. Furthermore, changes in extracellular Na^+ that cause a sustained elevation in $[Ca^{2+}]_i$ also decrease the respiratory burst. It is possible that changes in $[Ca^{2+}]_i$ involved in termination of the respiratory burst act through activation of PP2b.

In summary, we have extended our previous studies on the signaling pathways stimulated by ADP in rat AM to the potential role of PKC and serine–threonine

protein phosphatases. The ADP-stimulated burst is activated through signaling pathways that are largely dependent on PKC, in particular, isoforms that are inhibited by BIM. Furthermore, we showed that the protein phosphatases PP1 or PP2a and PP2b are involved in the termination of the ADP-stimulated respiratory burst; PP1/PP2a might also participate in activation of the respiratory burst enzyme. Further studies examining the phosphorylation of specific components of the respiratory burst oxidase may reveal the specific steps that are affected by the inhibitors used here.

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